RETROSPECTIVE FRAGILE X STUDY BY CAPILLARY AND AGAROSE GEL ELECTROPHORESIS

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ABSTRACT

Fragile X Syndrome (FxS) is the most common cause of inherited mental disability and autism spectrum disorder. Estimated incidence in males is 1 in 4000 and 1 in 7000 for females. The gene is on the distal end of the long arm of the X chromosome and contains a trinucleotide cytosine, guanine, guanine (CGG) microsatellite repeat in the 5' untranslated region (5'UTR) of the gene. When the repeat region is fully expanded the CpG island near the gene promoter site becomes methylated, and the result is silencing of the gene and loss of protein product. Health issues that correspond to a partially expanded trinucleotide repeat are Fragile X Tremor and Ataxia Syndrome (FXTAS) and Primary Ovarian Insufficiency (POI).

Current molecular detection involves Polymerase Chain Reaction (PCR) amplification of the CGG region, followed by capillary electrophoresis (CE) separation and fluorescent detection of the PCR amplicon with very high sizing resolution for normal and lower range premutation repeats. For larger pre-mutation repeats and full mutations, detection is done with digestion of genomic DNA with methylation sensitive enzymes, followed by Southern blotting. The Southern blots have some significant disadvantages; they are labor intensive, require large amounts of genomic DNA, blot sizing of the CCG repeat region has very imprecise resolution, reagents are expensive and they have a long processing time.

Fluorescent detection of PCR product by CE is faster, less expensive, and has

much higher resolution, but it has not been useful for the detection of full mutations due to PCR inefficiency for large CGG repeats and difficulty of detecting a very large CG rich trinucleotide repeat PCR product.

The purpose of this thesis is an overview of the current molecular paradigms of FxS, FXTAS and POI, molecular testing and a comprehensive evaluation of the Celera Fragile X assay and the possibility that use of their 1.5% agarose recipe, gel electrophoresis (GE) and a long injection capillary electrophoresis protocol can detect all premutation and full mutation samples and reduce the need for Southern blotting for molecular detection of full mutation patients.

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INTRODUCTION

Fragile X Syndrome (FXS) is the most common cause of inherited mental disability and autism spectrum disorder.[1, 2] In 1991 scientists discovered the gene *FMR1* (Fragile X Mental Retardation–1) that causes fragile X syndrome. The gene is on the distal end of the long arm of the X chromosome and contains a trinucleotide cytosine, guanine, guanine (CGG) microsatellite repeat region in the 5' untranslated region (5'UTR) region of the gene. It is evolutionarily conserved in many species, with orthologues in other vertebrates and drosophila.[1] Fragile X Mental Retardation Protein (FMRP) is an RNA binding protein and contains common RNA binding motifs: two K-homologous (KH) domains and an N-terminus RGG (arginine, glycine, glycine) box (see Figures 1 and 2).

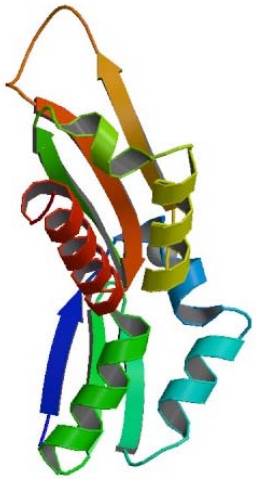

Figure 1. Fragile X Protein KH domains ribbon diagram

http://www.rcsb.org/pdb/home/home.do

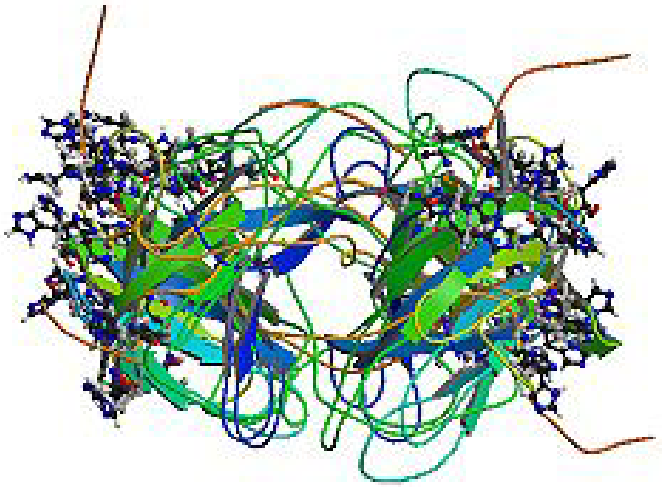

Figure 2. Fragile X N-Terminal RGG domains ribbon diagram

http://www.rcsb.org/pdb/home/home.do

The nuclear localization and export signals on the protein show that FMRP is involved in shuttling RNA to and from the nucleus.[3]

The molecular mechanism for the pathology related to FMR1 is expansion of the CGG region, with differing sizes of microsatellite repeats corresponding to different health and development issues. When the CGG repeat region is fully expanded (>200 repeats) the CpG island near the gene promoter site becomes methylated and the chromatin shifts from an active euchromatic, transcriptionally open confirmation to a heterochromatic, condensed nontranscribing state. [4] This methylation extends to the adjacent promoter region and the result is silencing of

the gene and loss of protein product. More than 95% of Fragile X Syndrome (FxS) incidence is caused by the expansion and resulting methylation of this repeat region, and 5% is caused by other gene anomalies, i.e, promoter region deletions or nucleotide mutations within the gene. Fragile X Tremor and Ataxia (FXTAS) and Primary Ovarian Insufficiency (POI) are diseases also related to the alteration of the CGG region size in this gene, but they have completely distinct molecular mechanisms from FxS.[5]

The size of the repeat region determines gene functionality and mitotic stability of the repeat region. Normal expression of the gene correlates to repeat sizes up to 45 CGG repeats. Between 45 and 55 repeats is considered a “grey zone” due to the uncertainty and variability of gene expression and the possibility of repeat expansion during transmission caused by mitotic instability related to haplotype sequence interspersions. The CGG repeat is normally interrupted by two stabilizing AGG repeat elements at positions 10 and 20. Alleles that lack the 3’ nearest AGG element are more likely to expand upon transmission, and loss of both stabilizing AGG elements increases expansion potential even more. Other haplotype markers used in allele expansion association studies are DXS548, FRAXAC1 and two dinucleotide markers (CA) 150 and 7Kb distant from the CGG repeat. [6] Regions with 55 to 200 trinucleotide repeats are classified as premutation (PM) and can be mitotically unstable during oogenesis, depending on both the repeat size and the haplotype sequence interspersions. A full mutation (FM) of 200-1000 repeats is associated with methylation of the promoter region, silencing of the gene and complete loss of protein expression.

FxS Prevalence

FxS is regarded as the most common form of inherited cognitive impairment and a prevalence estimate has been reported to be 1 in 4000 for males and 1 in 7000 for females, but this is not a consensus. [7] There are variations in estimates of its prevalence in the population. The most common estimates are based on population projections from special education needs children; this skews the estimates of FxS prevalence. There are likely many individuals who exhibit the behavioral, cognitive and learning disabilities associated FxS but have border line or near normal IQs, particularly females. Additionally, the studies from which these statistics are drawn from are often too small to be significant and do not consider possible regional founder effects. Paul Hagerman, professor at the Department of Biochemistry and Molecular Medicine, University of California, Davis, proposes using prevalence of PM alleles in the population from direct screening for large scale populations of normal pregnant or preconception women and the known relationship between PM alleles and the statistical frequency of expansion to FM allele. Using this method of prevalence estimate, there would be 1/2,500 FM in the population for both males and females.[7]

FxS Phenotypes

A fully expanded CGG repeat (>200 repeats) is associated with a complete loss of protein product. Affected males are always symptomatic with possible variable expression due to repeat size mosaicism and have developmental issues that present in early life and persist with a life-long behavioral phenotype. Patients affected with FxS exhibit intellectual disabilities with IQ's ranging from 20 to 70, speech and language impairment and autistic like behaviors, including anxiety, irritability, unstable moods and deficits in pre-pulse inhibition to stimuli, usually acoustic.[2, 8] In addition, 20% of affected patients experience seizures of either benign epileptic or audiogenic types.[9-11] The cognitive dysfunctions include deficiencies in working and short term memory, executive functions, mathematic and visual-spatial abilities.[12] The neuronal dendritic spines in the brains of FxS patients are immature, elongated and thin due to incomplete synaptogenesis.[13] Cognitive impairment appears initially mild in males but seems to become more severe with age. Females can have a less severe phenotype, due to the protective effect of random X chromosome inactivation.[14] The majority of full mutation females are affected cognitively with 70% demonstrating an IQ in the borderline or mentally retarded range. The remaining 30% of females have IQs in the normal range, but can have learning disabilities and emotional problems.[15]

Behavioral aspects vary with age and gender and can include autistic-like behaviors including poor eye contact, perseverative speech, tactile defensiveness, shyness, social anxiety, hand flapping and biting. Anxiety and

mood disorders, hyperactivity, impulsivity and aggressive behaviors are often present with varying severity.[13, 16]

In addition to incomplete neural development, cognitive impairment and behavioral hallmarks, there are several physical features associated with the FxS phenotype including craniofacial anomalies, i.e., a long face, a large prominent jaw, elongated or everted ears and close interocular distance. [1, 17] Patients may also exhibit flat feet, hyperextensible finger joints, hand calluses, strabismus and machroorchidism in males and enlarged ovaries in females. The representation of the physical hallmarks is similar in both males and females before puberty, with the proportion of females with physical hallmarks being slightly less in some features. Postpubertal females show the physical hallmarks about half as frequently as postpubertal males. [15]

FxS Molecular Basis

The syndrome name comes from the appearance of a chromosomal fragile site when cells with an expanded CGG region are cultured in a medium deficient in folic acid.[1] The *FMR1* gene codes for FMRP, an mRNA binding protein that appears to be a key player in neuronal synaptic plasticity through negative translational control and transport of mRNAs as a molecular chaperone.[18] When the repeat region is ≥200, the initial pathogenic event is the methylation of the CpG region in the 5'(UTR). Early germ cells from full-mutation fetuses have expanded repeats but are unmethylated, whereas cells taken from chorionic villi samplings from full-mutation fetus's show FMR1 hypermethylation

that increases in proportion to the degree of development. This demonstrates that the gene silencing happens during embryonic development and is a dynamic process. [4, 19, 20] The mechanism of this event is unknown but is widely recognized as the cause of gene silencing because of the existence of rare patients with full mutation repeat regions but no CpG methylation that do not exhibit the FxS phenotype and are not gene mosaics.[21, 22] This epigenetic change alters the structure of the gene. The methylated CpG residues are recognized by methyl binding proteins, and a multiprotein complex is recruited with histone deacetylases (HDACs) resulting in condensation of chromatin. This restricts access of polymerase and transcription factors to the promoter region preventing transcription. [23]

The loss of FMRP in dendrites and synaptic junctions has multiple downstream effects due to the fact that the protein is a chaperone of many mRNA's that are involved in synaptic functioning. The candidate downstream targets identified in this cascade include a significant percentage (>4%) of brain mRNAs, particularly those containing a G-quartet structure or a uracil rich sequence. [13]

The FMRP targets are involved in multiple critical neuronal systems, including cytoskeleton modeling, mRNA translation repression, and signal transduction. The loss of the protein impacts neuronal plasticity during development, preventing neuronal maturation and synaptic function.[8, 9] FMRP is cytoplasmic but has nuclear localization and nuclear export signals involved in shuttling the bound mRNAs into the nucleus. The nuclear proteins targeted are

involved in mRNA translation and/or dendritic transport inducing local protein synthesis in the vicinity of activated synaptic spines.[24]

Neurotransmitters (NTs), mostly glutamate, and neurotrophic factors such as brain derived neurotrophic factor (BDNF) activate synthesis of FMRP at synaptic junctions in normal patients. [20] The glutamate targets the mGluR5 metabotropic receptor and induces local synthesis of FMRP at the synapse. The presence of FMRP is an inhibitor of mGluR5 stimulation by negative feedback. Loss of the FMRP feedback regulation results in an increase in mGluR activation and a loss of inhibitory signaling in the neurons. FMRP is a negative regulator of transcription so in the neurons in FXS patients the absence of FMRP leads to an increase of the internalization of these ionotropic and metabotrophic glutamate receptors which results in excessive neuronal long term depression (LTD; long lasting decrease in synaptic connectivity) and weakening of synaptic connections.[25] LTD and LTP (long term potentiation) are known to be involved in learning and memory; this relates to the cognitive disabilities in FxS patients. The mGluR model is supported by a study where mice that are *Fmr1*(-/Y)*Grm5*(+/-) show substantial correction of deficits in neural plasticity and normalization of dendritic spine density.[13] The mechanisms behind the hyper-excitability leading to seizures are also linked to the mGluR model. Epileptogenesis is related to the failure to modulate the mGluR5 response in the absence of FMRP results in neuronal hyperexcitability. The generation of neuronal current reduces excitatory input to inhibitory neurons and overall increase in neuronal circuit excitability.[26]

Other perturbations in cellular signaling extend to gamma-amino-butyric acid (GABA) gated anion channels and to other non-mGluR G-protein-coupled receptors (GPCRs), including dopamine receptors and acetylcholine receptors.[27] GABAergic signaling is the major inhibitory neurotransmitter in the central nervous system (CNS). It is likely that FMRP related dysregulation of this system also contributes to increased neuronal excitatory behavior and seizures.[26]

FMRP plays an important role in expression of the structural proteins, microtubule-associated protein (MAP1B) and neuron-enriched acidic protein having a molecular mass of 22 kDa (NAP22) in the dendrites after specific triggering. Loss of FMRP causes abnormal synaptic protein synthesis that underlies the variable symptoms of FxS including the presence of immature spines and impaired synaptic maturation. In association with polyribosomes and microtubules, FMRP helps regulate the formation and pruning of dendrites.[13, 28]

Treatment FxS

Treatment for FxS patients is currently supportive and behaviorally targeted to maximize functioning because there are no FDA approved pharmacological strategies directed at the specific neuronal defect of FMRP absence. Symptom based treatment of the most problematic behaviors can be helpful. Standard treatment includes special education, speech, occupational, and sensory integration training and behavior modification programs. Surgical correction of heart defects is sometimes necessary.[29, 30] Table 1 describes the symptomatic medications that are current standard of care.

Table 1. Recommended medications for symptomatic treatment of FxS[15]

Symptoms	Medications
Seizures Mood Instability	Carbamazepine Valproic Acid Lithium Gabapentin Lamotrigine Topiramate Phenobarbital
Attention Deficit	Ritalin Adderall Effexor Amantadine Folic Acid
Hyperarousal Sensory over-stimulation	Clonidine Guanfacine
Aggression Intermittent explosive disorder Obsessive-compulsive disorder Anxiety Depression	Prozac Zoloft Celexa Paxil Luvox
Sleep Disturbances	Trazodone Melatonin

There is research currently in progress to identify pharmacological targets in the syndrome. The most interest is in the mGluR5 post synaptic glutamate receptor and the gamma-aminobutyric acid-ergic ($GABA_B$ergic) system. In a *Fmr1* knock out mouse, progress has been made towards rescue of behavioral and structural abnormalities with the mGluR5 post synaptic glutamate receptor antagonist fenobam, which reduces mGluR5 activation of downstream processes, as does Lithium.[17] A pilot, open label, single dose trial of fenobam showed the positive effects seen in animal models of FXS was replicated in 9 of 12 human subjects who had IQs ranging from 36-85. Improvements included improved prepulse inhibition, calmed behavior, increased eye contact, cognition

and interaction.[11, 13] Arbaclofen, a prodrug for the $GABA_B$ receptor agonist, Baclofen, has been shown to decrease the irritability and aberrant behavior seen in FxS and is also undergoing clinical trials. Aripiprazole is an antipsychotic that is being evaluated for FxS patients as well and could be effective in decreasing aggression, self-injurious behavior, agitation, and repetitive behavior commonly observed in individuals with FxS.[10, 15] FMRP replacement therapy is being studied in Fmr1 knock out mouse models of FxS. Scientists at University of Florida College of Medicine have constructed an adeno-associated virus vector that expresses the central nervous system isoform of FMRP. The fmr1 knock out (KO) mouse phenotype showed rescue from the long term depression that may be linked to cognitive impairments associated with FxS. Analysis of hippocampal synaptic function in these mice showed that the paired pulse low frequency stimulation induced LTD was restored to WT levels.[31]

FXTAS Prevalence

One in 813 males and one in 259 females of the general population carry a PM allele. Different ethnicities show different prevalence, the PM allele is less common in Asian populations, more common in Mediterranean groups. A recent study of 40,000 women in Israel showed 1/154 with the PM allele.[21] Penetrence and incidence of FXTAS, including age of onset and severity of symptoms, is variable and is directly related to the size of the premutation allele.[32]

FXTAS Phenotype

Premutation carriers with a CGG repeat region between 55 and 200 in FMR1 do not show fragile X syndrome phenotypes but can experience other serious health issues, usually late onset syndromes around 40 or 50 years of age or later. [9, 33, 34] The most common late onset disorder is Fragile X Tremor and Ataxia Syndrome (FXTAS). The syndrome was not described until about 10 years after the discovery of *FMR1* in 1991. Geneticists were focused on the developmental disorder associated with mental retardation. Pediatric clinics had little contact with aging relatives of patients with FxS so the age associated symptoms were not recognized as being related to carrier status. [35]

Clinical features of FXTAS include Parkinsonian like intention tremor, gait ataxia, peripheral neuropathy, cognitive decline beginning with memory deficits, psychiatric problems and autonomic dysfunction (high blood pressure, impotence, bladder/bowel control). FXTAS is observed in 40% of male carriers older than 50 years of age. It is also observed in females, although at a lower rate of incidence, again due to the protective effect of random X inactivation.[14] The penetrance of the phenotype increases with age and number of CGG repeats.[35-37] This makes FXTAS one the most common causes of tremor, ataxia and cognitive decline among older adults. [38]

Other clinical features include brain atrophy with white matter disease and characteristic hyperintensity in the middle cerebellar peduncles. The psychiatric problems, including schizoid, obsessive-compulsive, phobic and psychotic symptoms are likely due to neurodegeneration in the amygdala, as memory

deficits are due to the same in the hippocampal regions.[5, 39, 40]

The behavioral problems seen in FXTAS include significant deficits in ability to inhibit prepotent impulsive responses and impaired selective attention. In one study of PM males the inhibition deficits developed at age 30 onwards and severity of this behavior was seen as a neurological sign preceding cognitive deficits, tremor and ataxia to develop later in the PM male carriers' life.[17, 39, 41] It is generally accepted that premutation carriers have normal IQ until late onset of cognitive decline.[40, 42]

FXTAS Molecular Basis

Premutation carriers have 5-10 times the abundance of FMR1 mRNA due to a hyperactivated promoter region and possible negative feedback mechanisms, but slightly reduced abundance of the functional protein relative to normal patients.[43] The resulting RNA toxicity from mRNA over abundance is the accepted cause of FXTAS and several other trinucleotide repeat (TNR) diseases, but the exact mechanism for the RNA neurotoxicity is debated and there are many studies of the various downstream affects. The resulting dysregulation from the toxic gain of function mRNA affects a number of proteins as well.

In both mouse and drosophila models the FXTAS phenotype is observed when an expanded CGG repeat is transcribed upstream of an unrelated reporter gene.[35, 44, 45] The presence of RNA CGG repeats outside the context of *Fmr1* is sufficient to produce ubiquitin positive inclusions. This supports a theory that expanded CGG repeat regions form a short artificial hairpin structure that

resembles a heteroduplex. This structure can bind to and sequester RNA binding proteins. Additionally, ribonuclease Dicer cleaves long C(any nucleotide)G repeat regions into structures that resemble regular duplexes despite many basepair mismatches. [46] These duplexes may trigger cellular responses, like the RNA interference (RNAi) or micro RNA (miRNA) pathways, that down regulate transcription.[45, 47]

A different theory states that rCGG binds with and sequesters the proteins hnRNP and CUGBP1, that are involved in transcription, mRNA trafficking, splicing and translation. This theory is supported by experiments where over expression of these two proteins suppress Fragile X CGG PM repeat-induced neurodegeneration in a drosphilia model.[35, 48]

Another theory is that the excess rCGG repeat binds many other mRNA binding proteins that are involved in regulation of mRNAs involved in RNA splicing. The neuropathology of FXTAS includes the presence of ubiquitin and FMR1 mRNA positive nuclear inclusions in the neurons of affected patients. The presence of about 30 proteins has been observed by mass spectrometric analysis of neuronal inclusions. The size of the premutation repeat is directly proportional to the severity of the FXTAS symptoms, earlier age of onset and the proportion of inclusion bearing hippocampal and cortical neurons. [5, 35, 37, 47]

The protein FMRP is expressed in many tissues, but more highly in gonads and brain.[15] This organ specific expression might account for both the late onset premutation health issues linked to FXTAS neurological and POI phenotypes by mRNA toxicity and also the cognitive developmental issues in

FXS from gene silencing and lack of protein expression .[1, 35]

FXTAS Treatment

Currently, there are no targeted therapeutic interventions that affect the pathogenesis of FXTAS. The treatment approaches are symptom based and there are neuroprotective agents that may slow the course of FXTAS. The drugs used for treatment of the tremor are primidone, beta-blockers, benzodiazapines and memantine. Propranolol, a B-adrenergic blocker is the most effective treatment of tremor. Botulinium toxin injections have had some success with tremor as well. Deep brain magnetic stimulation has been reported on 3 patients with tremor with a marked reduction in tremor for about 4 months although gait ataxia persisted. Ataxia has shown improvement in patients treated with cabidopa/levidopa, dopamine agonists and eldepryl. Physical therapy can be useful for improving strength and gait. The treatment of related cognitive defects and dementia is based on off-label application of dementia treatments conventionally used in Alzheimer's disease (AD). Cholinesterase inhibitors are used for memory impairment. Psychiatric problems that occur prior to the onset of FXTAS can be treated with antidepressants and antipsychotics along with cognition enhancers. Autonomic dysfunction, such as urinary urgency and frequency has been treated with injections of botox into the lining of the bladder.[32]

Diet and exercise have been evaluated as treatment targets in Alzheimers Disease, dementia and related conditions. The nutritional deficiencies that impact

patients with FXTAS should be similar to other cognitive and movement related disorders. Deficiencies in B vitamins like folate and B12 are common in elderly patients and are associated with increased risk of cognitive decline and dementia. Hyperhomocysteinemia occurs with low folate or vitamin B12 and is associated with increased prevalence and incidence of AD and dementia. Both vitamins are involved in the synthesis of S-adenosylmethionine (SAM) which is a methyl donor for a variety of methylation reactions, including those involving neurotransmitters, membrane phospholipids, myelin and DNA. The B vitamin has been associated with depression which is also a symptom in FXTAS.[32]

POI Prevalence

Of the 1/259 female carriers of the PM allele and 20% have Primary Ovarian Insufficiency (POI), defined as amenorrhea or cessation of menstruation at or before 40 years of age, compared to 1 % in the normal population.[21, 35] An additional 20% of PM carriers show POI before the age of 45. [1, 35] There is a study that reports that POI preferentially affects women who inherited the premutation from their fathers, suggesting an imprinting phenomenon, but other studies failed to confirm these findings.[49] Full mutation carriers do not have POI; they have the same risk of ovarian dysfunction as noncarrier women.[1, 49, 50] Studies correlated the size of the premutation repeat region with ovarian dysfunction and a positive, nonlinear association with CGG repeat number and age at POI onset was found. Women with POI were more likely to have a midsize repeat region around 70. Women with PM regions over 100 were less likely to

have POI, perhaps due to skewed X inactivation related to larger premutation regions.[20, 49]

POI Phenotype

POI is the development of amenorrhea, with sex hormone deficiency and elevated serum gonadotropin levels before age 40. The ovaries stop functioning normally, sometimes with the permanent cessation of menses.[49] One group found that there was no difference in the occurrence of diseases known to be associated with menopause, such as cardiovascular diseases and osteoporosis in premutation patients with POI relative to normal patients. Premutation status may affect the ovaries, but does not substantially increase the risk for additional medical problems.[50] The fact that patients with full mutation alleles do not have POI supports that this phenotype is not related to fact that they have an average of 50% of cells that are devoid of FMRP. Women with the PM that are still cycling have higher levels of follicle stimulation hormone (FSH) than healthy women as well as elevated gonadotropin levels.[1, 19]

POI Molecular Basis

There are no reports for the mechanism of POI, although studies have shown that it is related to the toxic mRNA gain of function effect. Further investigations are needed to understand the molecular pathways underlying POF among PM carriers, including the role of increased FMR1 transcripts on follicular development. [19]

POI Treatment

Management of POI is directed at symptom resolution. Hormone replacement therapy with oestradiol and progesterone is necessary to protect bone mass. Fertility treatments are not shown to increase pregnancy rates for patients with POI so psychosocial counseling and alternate family planning are recommended to accompany the diagnosis.[51]

FMR1 Inheritance

Inheritance of Fragile X syndrome and the premutation associated disorders differs from other X linked diseases, due to the mitotic instability and possible expansion of the PM repeat region. Carrier men pass a premutation unchanged and stable to all of their daughters but none of their sons. Normal and grey zone alleles are more unstable when transmitted through males.[25, 52] Each child of a carrier woman has a 50% chance of inheriting the PM allele. It can be transferred in a stable and unexpanded form or, due to the instability of the premutation region during oogenesis, the allele can expand into a higher premutation or the full mutation range, and Fragile X syndrome. The Fragile X premutation can be passed silently down through generations in a family before novel expansion of the gene occurs and the syndrome affects a child. The larger a repeat region, the more likely it is to expand to a full mutation during meiosis, adding increasing genetic anticipation to the inheritance incidence. Expansion risk is also related to the presence of the two AGG triplets within the CGG region (usually at positions 10 and 20) that have a stabilizing effect during mitotic

replication. Loss of one or both of the AGG triplets increases expansion risk.[53-56] The smallest repeat to expand to full mutation in one generation was 59 repeats and had loss of both stabilizing AGG segments.[55] In the same study, for 664 pregnant women with partially expanded *FMR1* genes, the overall risk of full mutation among PM mothers that transmitted the PM allele is summarized in Table 2. The study showed no differences in repeat expansion with respect to gender of fetus.[55]

The proposed mechanism of expansion involves slippage at the replication fork. Unpaired bases form loops, which result in expansions or contractions depending on whether the loop is on the template strand or on the synthesis strand. Large expansions likely involve displacement synthesis of Okazaki fragments. The hairpin structures formed include Watson-Crick base pairing that tolerates many mismatches. The unusual secondary structures of these hairpins may stall polymerases, resulting in large expansions. Unlike some other TNR diseases like DM1, there is no repeat instability in somatic cells.[19, 20]

Table 2. Transmission percentage of FMR1 PM alleles as FM alleles.[55]

CGG Repeat size of maternal allele	% Expanded to full mutation (≥200 CGG)
55-59	3.7
60-69	5.3
70-79	31.1
80-89	57.8
90-99	80.1
100-199	≈100

Molecular Diagnosis

Because of the multigenerational expression and variable phenotypes of fragile X-associated neuropsychiatric illness, there is a prominent role for genetic testing and genetic counseling of patients and their relatives. Genetic testing can be confirmatory for both full mutation and premutation of the *FMR1* gene and is an essential component of the clinical evaluation for families of FxS patients. Gray zone results (between 45-54 CGG repeats) should be interpreted within the context of the family and clinical history because of the uncertainty of expansion risk and expression variability.[11]

Current molecular detection involves Polymerase Chain Reaction (PCR) amplification of the CGG region, followed by capillary electrophoresis (CE) separation and fluorescent detection of the PCR amplicon with very high resolution for normal and lower range premutation repeats. For larger premutation repeats and full mutations, detection is done with digestion of genomic DNA with methylation sensitive enzymes, followed by Southern blotting (SB). The Southern blots have some significant disadvantages; they are labor intensive, require large amounts of genomic DNA, reagents, long processing time, and expensive reagents, and blot sizing of the CCG repeat region has very imprecise resolution (see Figure 3).

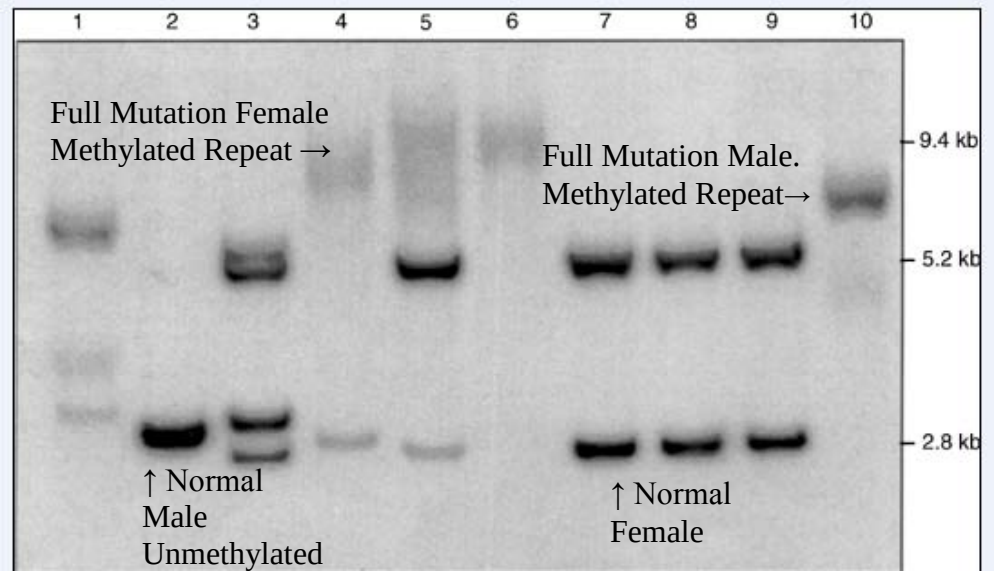

Figure 3. Southern Blot of 10 Patients. (www.currentprotocols.com/protocol/hg0905 accessed 12/13/2009)

Fluorescent detection of PCR product by CE is faster, less expensive, and has much higher resolution, but has not been useful for the detection of full mutations or large premutation alleles due to PCR inefficiency for large CGG repeats, preferential amplification of normal alleles and the difficulty getting large PCR fragments through the capillary. Apparent homozygous females and mosaics with PM/FM or normal/FM genotypes are not detected by CE so the SB is currently needed.

HYPOTHESIS/THESIS OVERVIEW

The Celera and Abbott Molecular fragile X research assay is a PCR assay that co-amplifies a gender-specific marker simultaneously with *FMR1* CGG triplet repeats up to 645 repeats, and sizes repeats up to 230 by fluorescent capillary electrophoresis (CE) on ABI ® 3130 Genetic Analyzer.

By using the Celera and Abbott Molecular Fragile X detection kit with CE and 1.5% Seakem and Nusieve agarose gels with UV detection, full mutation and premutations will be detected without Southern blotting. This study will determine whether or not Southern blotting can be eliminated from the Fragile-X detection protocol for the following genotypes: normal, premutation and full mutation females and males as well as mosaics. The Southern blot could be performed as a reflex on full mutation samples for confirmation of full mutation expansion and methylation status.

This study will attempt to answer the following questions;

- Will a combination of gel electrophoresis (GE) and CE be useful for detecting male full mutations, female full mutations and mosaics?
- Will a longer injection protocol on the 3130xl Analyzer improve detection of full mutations?
- Will the combination of the Celera gel detection and CE enable elimination of Southern blotting?

MATERIALS AND METHODS

Samples

Two hundred and fifty-nine premutation, full mutation and mosaic males and females from archived patient DNA were used for this study and amplified with the Celera and Abbot Molecular Fragile X kit. The DNA samples have been previously detected by PCR with either A.R.U.P.'s laboratory developed PCR assay with ABI 377 polyacrylamide slab gel fluorescent electrophoresis used from 2003-2006 or the Celera and Abbott Molecular kit and capillary electrophoresis using lab developed injection parameters. All samples were deidentified so it was not possible to distinguish which samples were detected with which methodology. All samples had been previously detected by Southern blot. Sample genotypes include CGG repeat regions from females and males with sizes in the grey zone (45-55), premutation zone (55-200), full mutations (200-1000) and genotype mosaics. The genotypes of the five mosaic patients were; female with three normal alleles, female with a full mutation, a premutation and a normal allele, male with two normal alleles, male with both premutation and full mutation alleles and male with two different sized full mutation alleles.

Instrumentation

Perkin Elmer 9700 thermocyclers were used for DNA target amplification by polymerase chain reaction with the Celera Abbot Molecular kit. The products were detected by fluorescent CE on the ABI 3130 genetic analyzer with both standard and long injections and run on Celera design lab made 1.5% Nusieve and Seakem agarose gels.

DNA Extraction

DNA was obtained from whole blood samples using the MagNA Pure ® System by Roche Diagnostics. It incorporates the use of magnetic silica beads, which allows DNA to bind to a silica surface to be transferred via magnetic charge. A total of 32 samples are processed in approximately 1 hour using the DNA I Fast Protocol. The process initially involves transferring samples into a 32-well sample cartridge. The cartridge is placed on the MagNA Pure along with all of the required reagents and disposable plastics. First, the MagNA Pure dispenses reagents into the respective areas in the processing blocks; lysis buffer is then added directly to the sample and mixed 1 row at a time. Next, the lysate is transferred to the processing block where it is dispensed into a row of wells filled with Proteinase K. Following room temperature incubation the samples are transferred to the next set of wells containing the magnetic silica beads. The DNA attaches to the beads and is then transferred via a magnet to wells containing wash buffer. The beads are subsequently washed and transferred (via magnet) to a sample cartridge that is preheated, allowing the

DNA to dissociate from the beads. The magnetic beads are disposed into the waste area and the elution buffer is transferred into the final sample cartridge, cooled at 4º C. One more disposal of beads occurs to eliminate any residue. Samples are then eluted for PCR amplification.

PCR Amplification

Extracted DNA was amplified by PCR using Celera and Abbott Molecular Fragile X ASR reagents. The master mix was prepared in a dedicated PCR area, free of amplicon. Table 3 indicates the volume of each reagent required for formulating the working master mix for a single PCR reaction and cycling conditions are described in Table 4.

Table 3. Reagent volumes for PCR amplification mix

Reagent Name	Volume for 1 PCR Reaction (uL)
High GC PCR Buffer	13
Gender Primers	0.6
Fragile X Primers	0.8
TR PCR Enzyme Mix	1.2
DNase/RNase Free Water	1.4
Total	17

Table 4. PE 9700 thermal cycling conditions for PCR reaction.

Temperature(℃)	Time (min:sec)	Cycles	Volume (uL)
98.5	0:10	15	20
58.0	1:00		
75.0	6:00		
98.5AutoX+0.1℃/cycle	0:10	15	
56.0	1:00		
75.0	6:00		
4.0	∞	-	

The master mix was vortexed and aliquoted into a 96-well plate. Three uL of extracted DNA was added to each well. The plate was centrifuged for 10 seconds at 500-2000x g to position the solution to the bottom of the tubes. The reaction was placed into a Perkin-Elmer PE 9700 thermocycler with the thermal profile in Table 4 and maximum ramp speed.

PCR Cleanup

Following PCR amplification, the product was put through a PCR product cleanup step in a postamplification area to remove unincorporated primers and dNTPs. In a new 96 well plate 3uL of CleanUp Enzyme provided by Abbott Molecular was added. Two uL of PCR product was added. The plate was centrifuged for 10 seconds at 500-2000 x g. The plate was placed in the thermal cycler with the Clean Up Program in Table 5.

Table 5. PCR cleanup PE 9700 thermal conditions.

Temperature	Time	Volume
75℃	10 min	5 uL
4℃	∞	

Capillary Electrophoresis

After the cleanup cycle the samples were prepared for capillary electrophoresis on the ABI 3130 Genetic Analyzer. The electrophoresis mix was prepared in a microcentrifuge tube as described in Table 6, for a total of n+2 reactions. The ingredients were vortexed prior to use. The electrophoresis mix was aliquoted to the 96-well electrophoresis plate and 5uL of cleaned PCR product was added. The plate was briefly centrifuged and then placed into the thermocycler for the denaturation program in Table 7.

Table 6. Electrophoresis reaction mix reagent names and volumes.

Reagent Name	Volume for 1 Reaction
Hi-Di Formamide	17.0 uL
ROX 1000 Size Standard	3.0 uL
Total	20.0 uL

Table 7. Denaturation thermal conditions.

Temperature	Time	Volume
93℃	30 sec	25uL
25℃	∞	

The samples were placed on the 3130 analyzer and injected with both a short injection protocol and a long injection protocol. The parameters of the injection modules are listed in Table 8. Fluorescent signal from the PCR product is detected by a CCD (charge coupled device) camera on the 3130xl analyzer, and the base-pair size determined by comparing the signal with the internal Genescan ROX 1000 bp size standard ladder by Genemapper ® analysis software.

UV Agarose Detection

The agarose gels used were made with the following protocol. As listed in Table 9, a 1.5 % gel mix was prepared in a 500mL flask. The agarose mix was heated in a microwave oven to dissolve agarose, then 10uL of 10mg/mL ethidium bromide was added and mixed by swirling the flask. The mixture was cooled in a water bath until slightly viscous. The solution was poured into a gel cast box and left to set for 1 hour at room temperature. Samples were prepared for gel loading with the following protocol in Table 10.

Table 8. Injection protocol parameters

3130xl Parameter	Short Injection Protocol	Long Injection Protocol
Oven Temperature	60°C	60°C
Poly_fill_Volume	6500	6500
Current Stability	5.0 uAmps	5.0 uAmps
Pre Run Voltage	15.0 kV	15.0 kV
Pre Run Time	180 sec	180 sec
Injection Voltage	2.0 kV	10.0 kV
Injection Time	2 sec	22 sec
Voltage Number of Steps	30 nK	30 nk
Voltage Step Interval	15 sec	15
Data Delay Time	120 Sec	120 Sec
Run Voltage	8.5 KV	8.5 KV
Run Time	2700 Sec	4000 Sec

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Table 9. 1.5% agarose gel mixture volumes and components.

Components	Amount
Molecular Grade Biology Water	180mL
NuSieve GTG Agarose	2g
SeaKem GTG Agarose	1g
10X Tris Borate EDTA(TBE) Buffer	20 mL
Total Volume	200 mL

Table 10. Agarose gel loading preparation.

Components	Volume
PCR Product	10uL
6X Loading Dye Solution	2 uL
Total	12 uL

The preparation for the gel size standard was prepared as described in Table 11. It contained DNA GeneRuler bp Ladder Plus (see Figure 4).

Limitations

Many of the archived DNA samples were degraded as shown by PCR failure of the gender control marker and subsequent analysis of a UV smear signal consistent with degraded genomic DNA with agarose UV detection.

Sample replicates for all samples were not possible, as sample DNA volumes were limited. The gels were very soft, fragile and difficult to load product on. Many of the poor UV signals were due to gel tears and reloading was not an option. The low agarose fluorescent signals required 10uL of PCR product per load. PCR product volume was 20uL and 2uL minimum volume was needed for CE. The 3130xl also has a high incidence of failed injections, so additional PCR product was occasionally needed to reinject.

Table 11. DNA GeneRuler bp Ladder preparation for agarose gel.

Components	Volume
DNA Ladder	1.0 uL
6X Loading Dye Solution	7.0uL
Molecular Grade Biology Water	4.0 uL
Total	12.0 uL

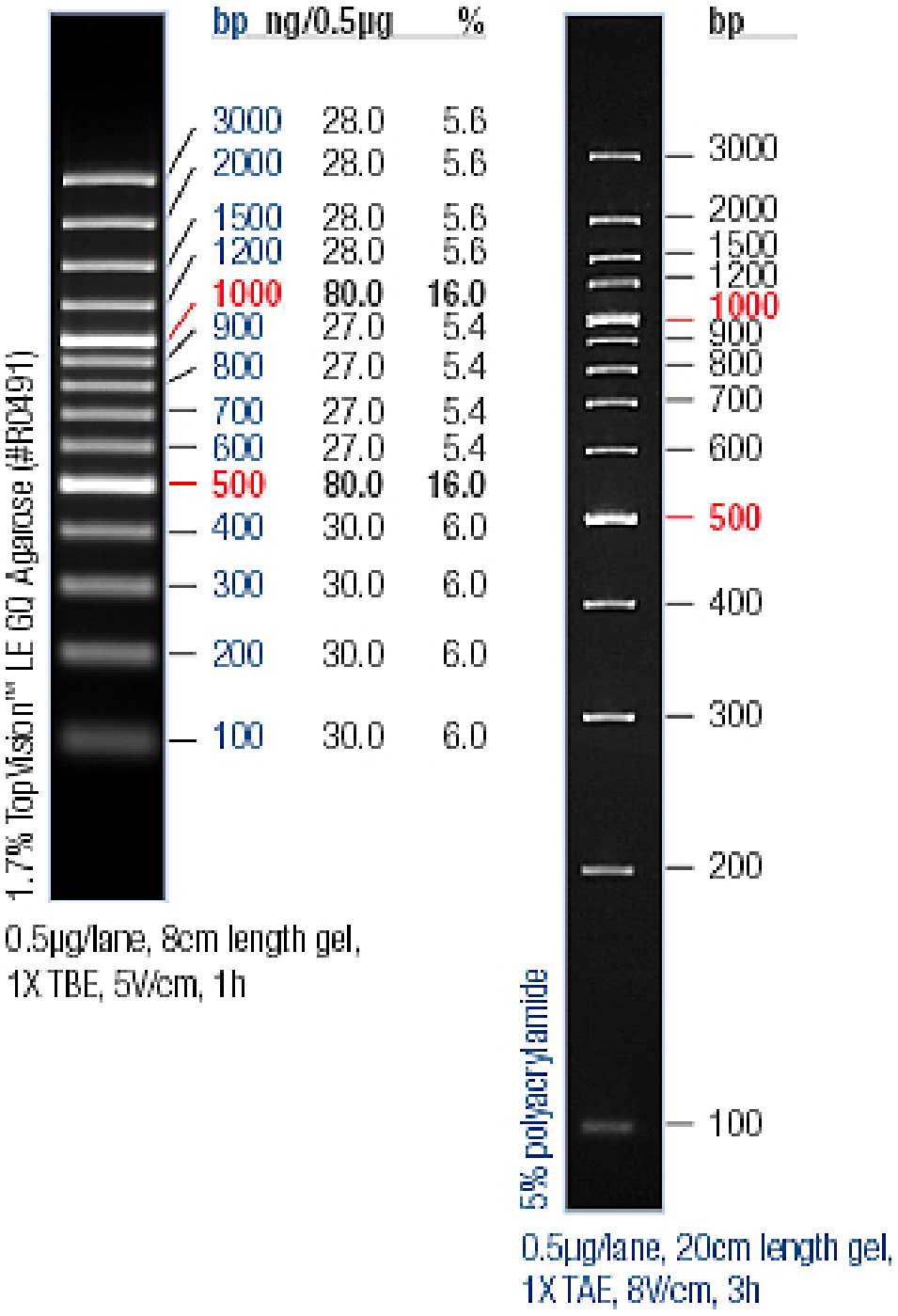

Figure 4. GeneRuler™ 100 bp Ladder plus base pair sizes.

RESULTS

Patient Samples

Of 259 samples 22 would not amplify for gender marker control or tri-nucleotide alleles. This is likely due to DNA degradation from extended frozen storage of samples. Samples that had amplification of the gender marker, but no amplification for the CGG repeat were not considered degraded. These samples were repeated and the gels checked for smear characteristic of degraded genomic DNA. One sample had no DNA volume in the sample tube. No results were obtainable for these samples.

Fourteen samples showed genotypes of gender and/or allele size that did not match their labels. Due to the many stages of labeling in the storage and de-identification process, it is likely these samples were mislabeled. Samples were repeated to confirm. Since the labels obviously didn't match the genotypes (i.e., gender mismatch, alleles clearly not matching the label) these samples are not included in the results. The labeled and actual genotypes are listed in Table 12. Table 13 shows the breakdown of samples that had results, or were mislabeled, degraded or absent.

Table 12. Mislabeled samples with label genotype and detected genotype.

Sample #	Label genotype	Genotype detected
16	M400	F30
57	M530	M27
73	M45	F45/30
78	F600/26	F72/26
85	F500/30	F57/29
98	M67	F66/30
107	M260/30	M no detection
108	F45	F44/30
124	M47	F25
125	F210	M46
127	F750	F74
154	F209/45	F29/45
171	F102	F98/30
236	F260	F30

Table 13. Samples that were tested, mislabeled, degraded or absent.

Total samples tested	259
Total samples mislabeled	14
Total samples with DNA Degradation	22
Samples with no DNA	1
TOTAL SAMPLES WITH RESULTS	222

Long Injection Protocol

The DNA samples were from archived DNA, some of the samples are several years old. This resulted in some samples not being amplified at all and others having some amplification, but very low signal. All of the samples were injected with a short injection protocol and a long protocol in order to ascertain whether this would enable detection of full mutations or large premutations. The long injection protocol was not useful for detection of large repeats, but some of the samples with very low amplitude on the short injection protocol resolved with better amplitude with the long injection protocol. Out of 16 samples with very low amplitude (<100) on the allele of interest, 4 resolved with ≥40% greater peak amplitude. Figure 5 shows example electropherograms (EP) of a sample that resolved with better amplitude.

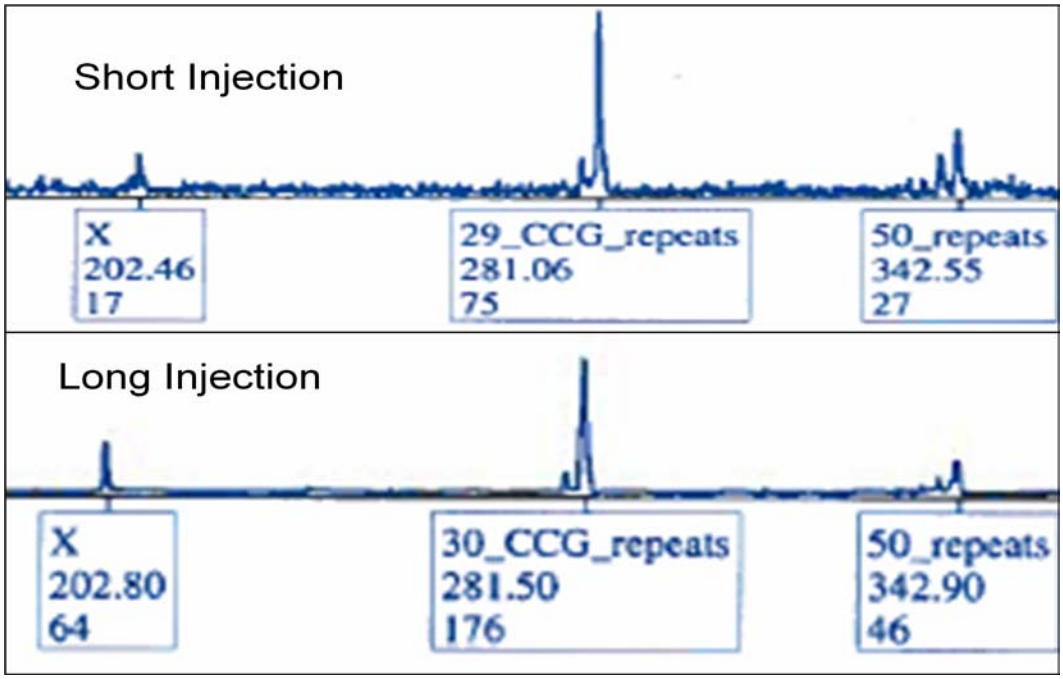

Figure 5. Resolution of low peak amplitude with long injection.

The FX alleles increase from 75 fluorescent units to 176 for the 29 repeat allele and an increase from 27 fluorescent units to 46 for the 50 repeat allele.

Samples with a robust PCR amplification and detection actually showed a drastic loss of peak amplitude with the long injection and a noisy peak. This could be due to irregular current patterns caused by too much product in the injection mixture in the system impeding the capillary flow. High salt concentration in the injection mixture disturbs the electrokinetic injection. Figure 6 shows EP where the peak amplitude decreased with the long injection protocol.

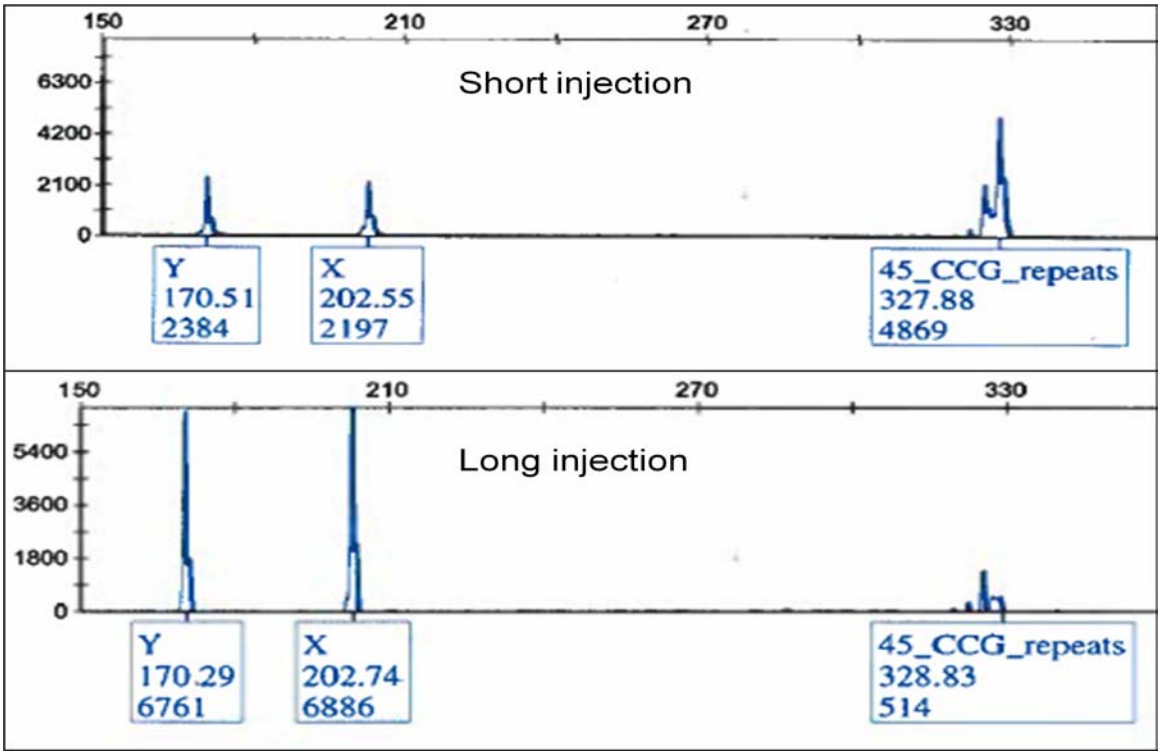

Figure 6. Loss of peak amplitude for long injection. The peak amplitude drops from 4869 fluorescent units to 514 for the FX allele and the peak is distorted on the electropherograms (EP).

Evaluation of Capillary Artifact

Of all the full mutation samples with good amplification for gender marker control, 17/23 females and 38/43 males had a fluorescent artifact within the 825 bps region or 205-211 repeats. This artifact is seen on most of the short injections and none of the long injections. The high number of full mutation samples with good amplification that had the artifact would suggest that the presence of this artifact implies full mutation product is present and is giving signal at this range of basepairs. This artifact was not seen on any of the samples where CE showed no product and was present in one premutation male. It was smaller in amplitude in female full mutations with a normal allele, which is to be expected with the shorter, normal alleles preferentially amplifying. Figure 7 shows examples of the artifacts appearance on 3 out of the 56 full mutation patient electropherograms.

Assay Sensitivity and Specificity

The genotypes and the number detected for male FM and PM, female FM and PM, and mosaics are listed in Table 14. The sensitivity of the assay for each genotype is listed in Table 15 for all the genotypes tested and includes samples that resolved with the long injection protocol. The specificity for all the genotypes tested by all three methodologies-CE alone, CE with the 211 artifact and with CE, 211 artifact and UV-is greater than 98.46% and each genotype is listed in Table 16.

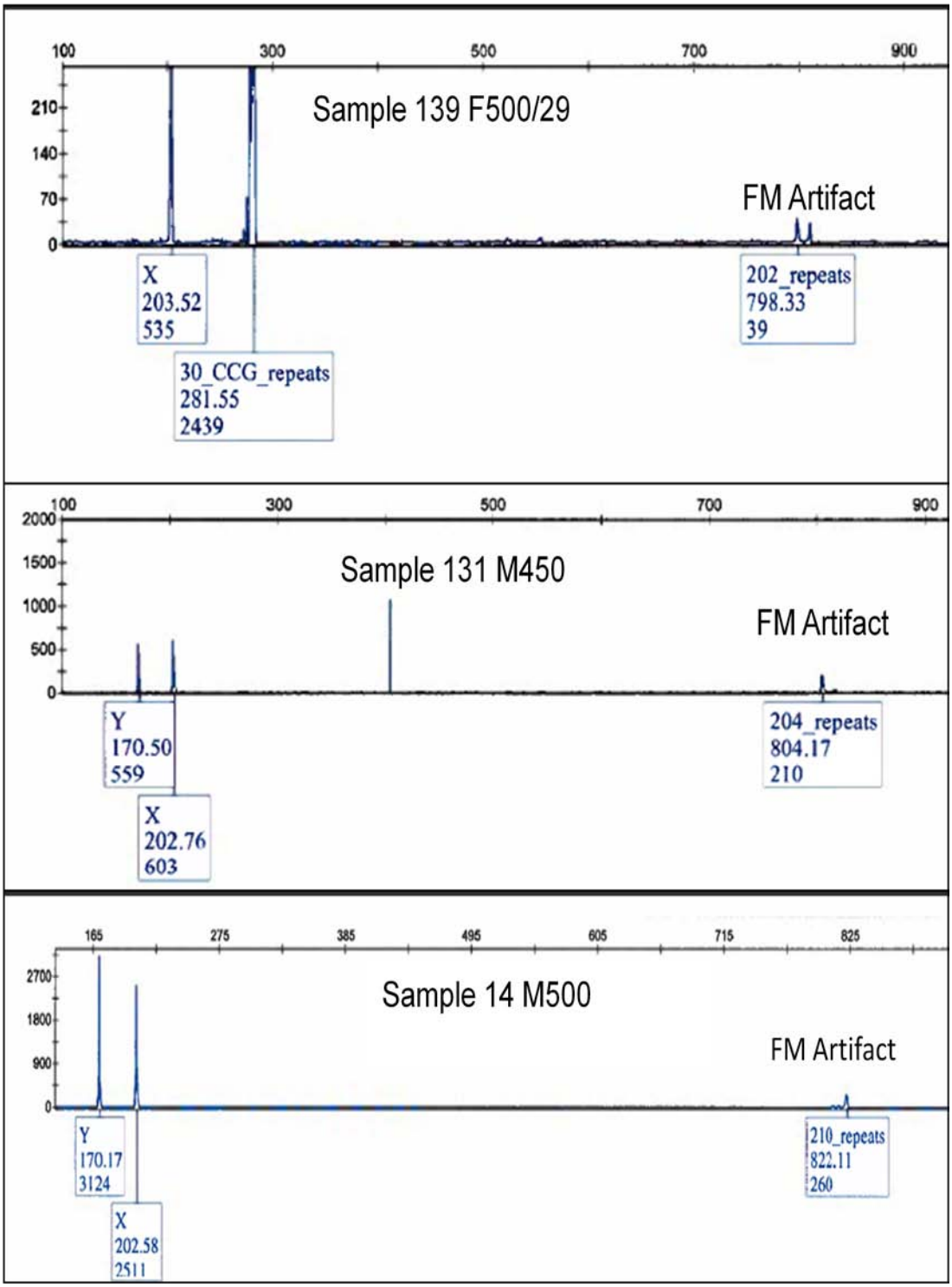

Figure 7. 211 artifact electropherograms for 3/56 detections

Table 14. Patient sample genotype detected by CE, CE + 211, CE+211+ UV

	Number of samples	Detected by CE	Detected by CE + 211 artifact	Detected by CE + 211 artifact + UV gel
Male FM	43	0	38	39
Female FM	23	0	17	17
Male PM	65	65	65	65
Female PM	86	85	86	86
Mosaic Female 3 Normal alleles	1	1	1	1
Mosaic Female 2 Normal and 1 FM allele	1	0	1	1
Mosaic Male 2 Normal alleles	1	1	1	1
Mosaic Male 2 different size FM alleles	2	0	2	2

Table 15. Sensitivity (True positives/ True positives + False negatives) % for male FM, female FM, male PM, female PM, Female normal mosaics, female FM mosaics, male normal mosaics, male FM mosaics.

	Number of samples	Sensitivity by CE	Sensitivity by CE + 211 artifact	Sensitivity by CE + 211 artifact + UV
Male FM	43	0	88.37%	90.70 %
Female FM	23	0	73.91%	73.91%
Male PM	65	100%	100%	NA
Female PM	86	98.84%	98.84%	NA
Female Mosaic Normal	1	100%	100%	NA
Female Mosaic Full Mut	1	0	100%	100%
Male Mosaic Normal	1	100%	100%	100%
Male Mosaic Full	2	0	100%	100%

Table 16. Specificity (True negatives/ True negative + False Positives) % for male FM, female FM, male PM, female PM, Female normal mosaics, female FM mosaics, male normal mosaics, male FM mosaics.

	Number of Samples for each genotype	Specificity by CE	Specificity by CE + 211 artifact	Specificity by CE + 211 artifact + UV
Male Full Muts	43	100%	100%	100%
Female Full Muts	23	100%	100%	100%
Male Pre muts	65	100%	98.46%	98.46%
Female Pre	86	100%	100%	100%
Female Mosaic	1	100%	100%	100%
Female Mosaic	1	100%	100%	100%
Male Mosaic	1	100%	100%	100%
Male Mosaic Full	2	100%	100%	100%

One premutation sample showed the 211 artifact bringing the specificity to 98.46%. This could be due to a small population of full mutation cells not detected by the Southern blot.

Largest Trinucleotide Repeats Detected

The largest PCR product detected by CE alone was a male PM with 147 repeats. It was detected at 139 by the electropherogram; the 8 repeat difference from the label could mean that this sample was originally run on the ARUP home brew assay that was in use previous to the Celera assay. The largest product detected by CE + 211 artifact was a male FM 1000. A summary of the largest product detected for large FM alleles and high PM/low repeat FM alleles is in Table 17. Figure 8 is the electropherogram of the largest capillary product detected and Figure 9 shows the largest UV Product seen.

Table 17. Largest repeats detected by CE, CE+ 211, CE + 211+ UV gel.

Genotype	CE	CE + 211 artifact	CE + 211 artifact + UV gel
Full Mutation males	N/A	1000	1000
Full Mutation Female	N/A	950	950
Pre-mut/ low FM male	147	N/A	200
Pre-mut/ low FM female	130	N/A	210

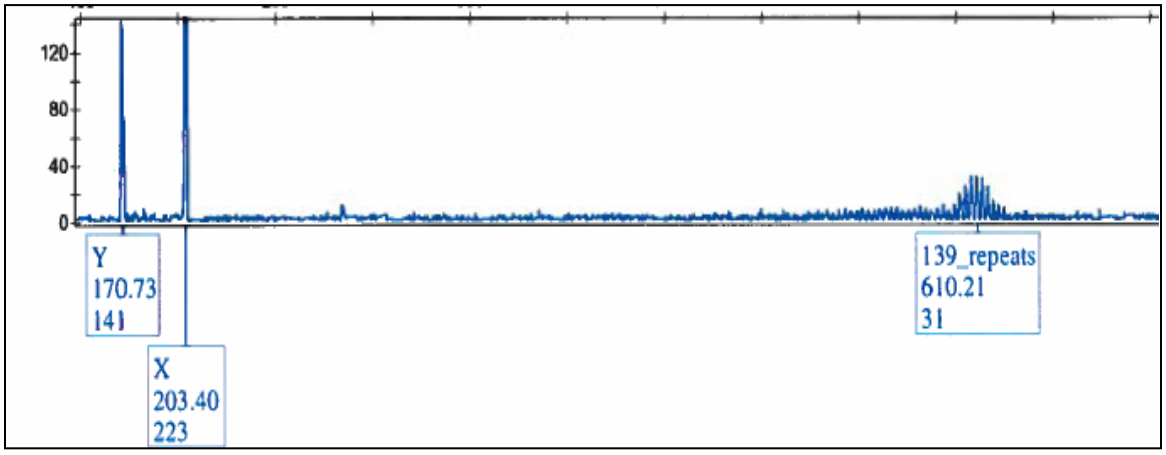

Figure 8. Electropherogram of largest allele detected on CE.

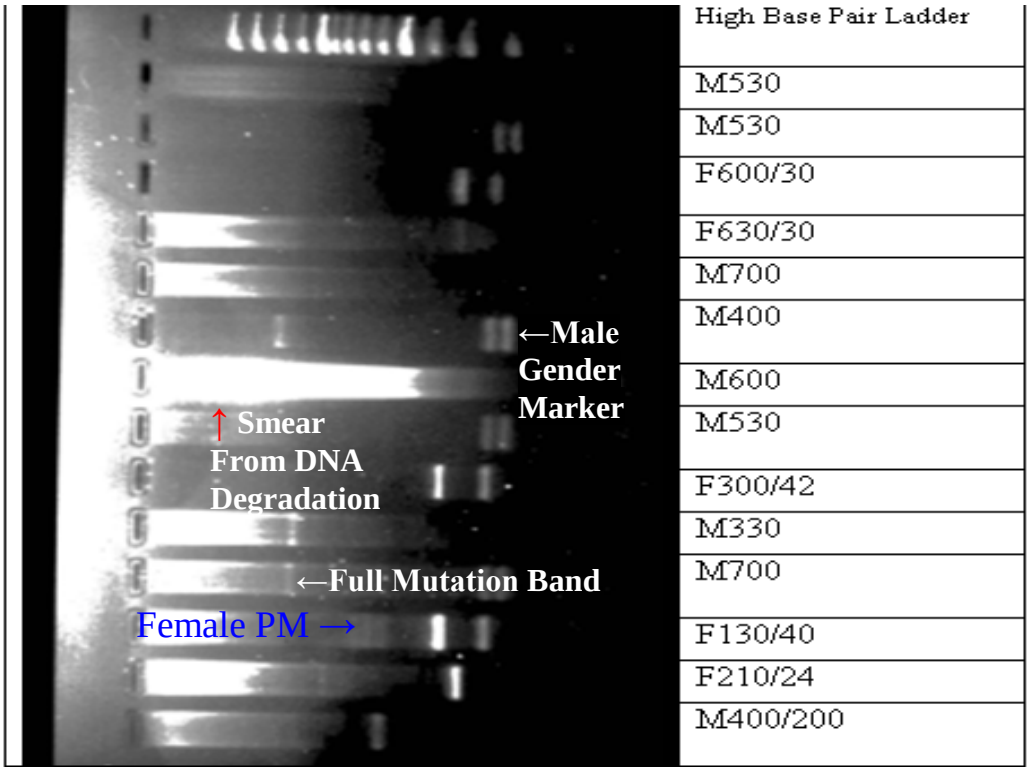

Figure 9. UV agarose signal of largest product detected by UV.

DNA Quality Evaluation and Second DNA Sample Set

To evaluate whether the poor quality of the archived DNA was affecting the PCR amplification and detection of the full mutation regions, a second set of DNA from a smaller, more recently extracted set of 17 deidentified full mutation patients was amplified. One was degraded and 4 were mislabeled. The labeled genotypes and detected genotypes are in Table 18. All 12 of the nonarchived samples showed the 211 artifact and had better results on the UV gels as well. Table 19 shows the number of samples detected by CE, CE with UV and CE by 211 artifact.

Table 18. Mislabeled samples from recently extracted DNA set.

Sample #	Label genotype	Genotype detected
273	F500/30	M full mutation
274	M600	F30/full mutation
276	F600/35	M full mutation
277	M630	M63

Table 19. Sensitivity for second set on nonarchived FM DNA.

Genotypes	Number of Samples	Sensitivity by CE	Sensitivity by CE + 211 artifact	Sensitivity by CE + 211 artifact + gel
Female Full Muts	3	0	3 (100%)	3 (100%)
Male Full Muts	9	0	9 (100%)	9 (100%)

DISCUSSION

With the prevalence of FxS and the recent discoveries about the pathogenesis and prevalence of the premutation disorders the molecular detection paradigm of this disease becomes increasingly relevant. The high sensitivity and specificity of this assay suggests that it could be an effective tool not only for FxS, FXTAS and POI diagnosis but for carrier or newborn screening. Screening for *FMR1* status has multiple applications: testing of pregnant females for potential fetal genotype, informing prepregnancy family planning or for premutation related disorder potential. However, there are ethical, legal and social concerns that are emerging due to the complexity, unpredictability and variability of *FMR1* inheritance and the broad expression of phenotype for all three *FMR1* disorders. The American College of Medical Genetics (ACMG) has not recommended *FMR1* for screening because of a lack of a cost effective test, the absence of data that there are benefits from screening and the possibility of negative effects.[57] The potential negative effects are myriad, and impact not only the mother and child, but other family members on social and medical levels. Identifying premutation carriers or full mutation patients gives implied genotype status for other family members without their consent or appropriate education on the genetic issues related to *FMR1.* There are studies that support the idea that early supportive therapy can be a benefit for FxS patients and families but there

are no data suggesting that this is true for FXTAS or POI. [10] The broad variability in expression could result in patients with mild, high functioning phenotypes being harmed by a diagnosis. Other genetic abnormalities, such as Klinefelter and Turner syndrome, could be detected with this assay as well.[57] To determine the accurate risk for pregnant carriers with premutation alleles passing a full mutation form of the allele they would need additional haplotype analysis and determination of the status of the stabilizing AGG elements in the CGG repeat. There are no commercially available tests for this information currently. With these complications, it is unlikely that there will be resolution on the appropriateness of prenatal or new born screening by the ACMG or other relevant parties soon.[58]

The purpose of this experiment was to evaluate the ability of the Celera kit to detect full mutation, premutation and mosaic samples both male and female using a combination of the regular CE protocol, a long injection protocol and the homemade 1.5% gels with the idea that Southern blotting could be eliminated from the current molecular diagnostic procedure.

The major findings were that no full mutation alleles were seen by CE with fluorescent detection without considering the FM fluorescent artifact. The detection rate for those full mutation samples by CE without the 211 artifact with UV detection was 0% for female full mutation samples and 44% for male full mutations. The detection rate using the CE + 211 + UV detection for male FM alleles was 90.70% and for female FM alleles was 73.9%. This could be used as

a qualitative indicator that the sample should be reflexed to Southern blotting, but is not informative for actual repeat region size.

This supports the hypothesis that Southern blotting could be eliminated for normal males, low premutation samples and normal female heterozygotes. Female homozygotes, male and females with large premutation alleles or full mutations or possible mosaic samples should still be reflexed to Southern blotting after evaluation of the electropherograms for a 211 artifact, and the UV gel for the presence or absence of a larger allele. This could decrease the processing time and expense for the majority of patients without increasing the risk of not detecting full mutation alleles.

The long injection protocol did not enable detection of larger premutation signals or any full mutations. The 211 artifact did not show up in any of the long injections. The longer injection was useful to boost amplitude on some samples with very poor amplification and normal to low premutation genotypes.

These findings are in concordance with the claims of Celera, Abbott Molecular and their collaborators from Oregon Health and Science University in the poster session at the 2006 ACMG meeting.[59] They have published that the largest repeat detected is 230 repeats and this study has the largest CE allele at 147. It is possible that there is some confusion that the capillary 211 artifact is actual signal. At the 2009 AMP meeting, Asuragen had a poster that showed the same 211 artifact. They call it a compression product and state that its presence is diagnostic for a full mutation. [60]

The experiment could be improved with better quality agarose gels. The Celera recipe made a gel that was very fragile and gave a less intense UV signal than gels available commercially. It is not clear that the 1.5% agarose concentration or the combination of Nusieve and Seakem agarose type is essential for detection and sizing of large alleles. It is also possible that changing the polymer type and the injection protocols could mitigate the conditions that caused the compression and/or lack of migration of the full mutation PCR product and boost detection rate for larger repeat alleles. More research into the repeatability of the 211 artifact with higher quality genomic DNA needs to be done to reflect the conditions that would be relevant in the clinical lab.

In summary this study demonstrated that the Celera and Abbott Molecular kit, combined with use of the 211 capillary artifact, and agarose gels with reflex to Southern blotting for female apparent homozygotes, patients with large premutation repeat regions and possible mosaics is an effective testing method with low risk of nondetection of patients with full mutations or mosaicism. The long injection protocol does not enable detection of full mutation or large premutation alleles, but can be useful for resolving samples with poor amplification.

With these findings it is perhaps time to reassess the ACMG's current recommendations about Fragile X screening. There are three screening applications that are being considered by the genetic community; carrier testing in pregnant or prepregnant females, newborn screening in males and females, newborn screening for methylated alleles. This assay gives no information about

methylation status but could be used effectively for the carrier testing or the newborn screening. With the specificity being almost 100% for all genotypes and the sensitivity for male full mutations being 90% it would be useful for screening newborn males. The sensitivity for females was 73% for full mutations and 98% for premutation carriers. This makes it less useful for screening full mutation female newborns, but an excellent method to detect pregnant or prepregnant carriers. With the complexity of the *FMR1* related transmission and phenotypes, the screening algorithm needs much consideration and discussion, but that conversation can be enabled by the improved detection methods discussed in this project.

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